CD73-Deficient Mice Have Increased Antitumor Immunity and Are Resistant to Experimental Metastasis

John Stagg, Upulie Divisekera, Helene Duret, Tim Sparwasser, Michele W.L. Teng, Phillip K. Darcy, and Mark J. Smyth

Abstract

CD73 is a cell-surface enzyme that suppresses immune responses by producing extracellular adenosine. In this study, we employed CD73 gene-targeted mice to investigate the role of host-derived CD73 on antitumor immunity and tumor cell metastasis. We found that CD73 ablation significantly suppressed the growth of ovalbumin-expressing MC38 colon cancer, EG7 lymphoma, AT-3 mammary tumors, and B16F10 melanoma. The protective effect of CD73 deficiency on primary tumors was dependent on CD8+ T cells and associated with an increased frequency of antigen-specific CD8+ T cells in peripheral blood and tumors and increased antigen-specific IFN-γ production. Replicate studies in bone marrow chimeras established that both hematopoietic and nonhematopoietic expression of CD73 was important to promote tumor immune escape. Using adoptive reconstitution of T regulatory cell (Treg)–depleted DEREG (depletion of regulatory T cells) mice, we demonstrated that part of the protumorigenic effect of Tregs was dependent on their expression of CD73. CD73-deficient mice were also protected against pulmonary metastasis of B16F10 melanoma cells after intravenous injection. Unexpectedly, we found that the prometastatic effect of host-derived CD73 was dependent on CD73 expression on nonhematopoietic cells. CD73 expression on nonhematopoietic cells, most likely endothelial cells, was critical for promoting lung metastasis in a manner independent from immunosuppressive effects. Notably, in vivo blockade of CD73 with a selective inhibitor or anti-CD73 monoclonal antibody significantly reduced tumor growth and metastasis of CD73-negative tumors. Taken together, our findings indicate that CD73 may be targeted at multiple levels to induce anticancer effects including at the level of tumor cells, Tregs, and nonhematopoietic cells. Cancer Res; 71(8); 2892–900. ©2011 AACR.

Introduction

The release of extracellular ATP by phagocytes and injured or stressed cells acts as a proinflammatory coactivator of the inflammasome via P2x7 receptor signaling (1, 2). In contrast, the phosphohydrolysis of extracellular ATP into adenosine, the mechanisms contributing to the immunosuppressive effects (8). We have recently demonstrated that one of the mechanisms contributing to the immunosuppressive accumulation of extracellular adenosine in tumors is the enzymatic activity of CD39 (NTPDase I) and CD73 (ecto-5′-nucleotidase), acts as an immunosuppressive pathway through the activation of adenosine receptors (3–6). In the context of cancer, landmark studies by Sitkovsky and colleagues have demonstrated that accumulation of extracellular adenosine in tumors suppresses antitumor immune responses, essentially via the activation of A2A adenosine receptors (7, 8). Extracellular adenosine levels are generally constant in most tissue but can rapidly increase in response to hypoxia and chronic inflammation (9). The process of extracellular adenosine accumulation in solid tumors has been recently reviewed (3). The accumulation of extracellular adenosine in tumors generates an immunosuppressive microenvironment that effectively enhances tumor immune escape. Activation of A2A adenosine receptors on T cells has been shown to inhibit T-cell–mediated cytotoxicity, cytokine production (10) and T-cell proliferation (11, 12) and to promote T-cell anergy (13).

The importance of A2A adenosine receptors in regulating antitumor immunity was first revealed by Ohta and colleagues who demonstrated that mice genetically deficient in A2A adenosine receptors spontaneously rejected immunogenic tumors in a mechanism involving increased CD8+ T-cell responses (8). We have recently demonstrated that one of the mechanisms contributing to the immunosuppressive accumulation of extracellular adenosine in tumors is the enzymatic activity of CD39 (NTPDase I) and CD73 (ecto-5′-nucleotidase), acts as an immunosuppressive pathway through the activation of adenosine receptors (3–6). In the context of cancer, landmark studies by Sitkovsky and colleagues have demonstrated that accumulation of extracellular adenosine in tumors suppresses antitumor immune responses, essentially via the activation of A2A adenosine receptors (7, 8). Extracellular adenosine levels are generally constant in most tissue but can rapidly increase in response to hypoxia and chronic inflammation (9). The process of extracellular adenosine accumulation in solid tumors has been recently reviewed (3). The accumulation of extracellular adenosine in tumors generates an immunosuppressive microenvironment that effectively enhances tumor immune escape. Activation of A2A adenosine receptors on T cells has been shown to inhibit T-cell–mediated cytotoxicity, cytokine production (10) and T-cell proliferation (11, 12) and to promote T-cell anergy (13).

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expression of CD73 by tumor cells (14). CD73 is a glycosylphosphatidylinositol (GPI)-linked cell-surface enzyme constitutively expressed on endothelial cells, Foxp3− T regulatory cells (Treg) and subsets of leukocytes, and is considered as the rate-limiting enzyme in the production of extracellular adenosine (15, 16). CD73 expression is induced by HIF-1α (hypoxia-inducible factor) activation, exposure to type I IFNs as well as activation of Wnt signaling (17, 18). CD73 is found expressed in several types of cancer (3) and has been associated with increased glioma cell proliferation (19). Intriguingly, CD73 expression is negatively regulated by estrogen receptor (ER) signaling (20). As a result, breast cancer cells deficient in ER signaling express high levels of CD73. We have recently demonstrated that CD73 expression on ER-negative mammary carcinoma cells significantly suppresses adaptive antitumor immunity and promotes tumor cell metastasis. Furthermore, we demonstrated that tumor-derived CD73 could be targeted for therapy with an anti-CD73 monoclonal antibody (mAb; ref. 14). Another independent group has since corroborated our findings in a mouse model of ovarian cancer, confirming that CD73 expression on breast and ovarian tumor cells promotes tumor immune escape (21).

While the immunosuppressive effects of CD73 expression on tumor cells have recently been studied, the effects of host-derived CD73 on antitumor immunity and tumor cell metastasis have not been investigated. In the non-hematopoietic compartment, CD73 is mainly expressed on endothelial cells and high endothelial venules (HEV), where it regulates leukocyte extravasation in peripheral organs (22) and inhibits inflammation-induced lymphocyte migration into draining lymph nodes, respectively (22). In hematopoietic cells, CD73 is most highly expressed on Foxp3+CD4+ Tregs and contributes to their immunosuppressive functions (11, 23). CD73 can also be found on effector T and B cells at lower expression levels.

We here report that CD73-deficient mice are significantly protected against the development of subcutaneous tumors and experimental lung metastases. We demonstrated that CD73 expression on nonhematopoietic and hematopoietic cells significantly restricts CD8+ T-cell–mediated antitumor immunity. Most importantly, we provide evidence that CD73 expression on Tregs is important for Treg-mediated tumor growth. Unexpectedly, we observed that CD73-deficient mice were significantly protected against the development of B16F10 lung metastases in a mechanism that was independent of CD73 expression on hematopoietic cells and independent of its immunosuppressive effects. We here propose that targeted inhibition of host-derived CD73 can enhance antitumor immune responses by inhibiting Treg-mediated tumor growth and prevent the development of distant metastases.

Materials and Methods

Cell lines, mice, and antibodies

All mice were bred and maintained at the Peter MacCallum Cancer Centre. EG7, MC38-ova, AT-3, and B16F10 mouse tumor cell lines have been previously described (24–27) and were authenticated by flow cytometry before use. C57BL/6 CD73-deficient mice were kindly provided by Dr. Linda H. Thompson (Oklahoma Medical Research Foundation, Oklahoma City, OK). C57BL/6 DEREG (DEpletion of REGulatory T cells) transgenic mice were kindly provided by Dr. Tim Sparwasser (Institute of Infection Immunology, Hannover, Germany). CD73 expression was assessed using phycoerythrin (PE)-conjugated anti-mouse CD73 mAb (clone TY/23; BD Bioscience), PE-conjugated anti-mouse CD45.1 (A20), fluorescein isothiocyanate (FITC)-conjugated anti-mouse Gr1 (RB6-8C5), Pacific Blue–conjugated anti-mouse CD4 (L3T4), PE-conjugated anti-mouse CD25, and allophycocyanin (APC)-conjugated anti-mouse CD8 (53-6.7) were purchased from BD Bioscience; FITC-conjugated anti-mouse CD11b (M1/70), FITC-conjugated anti-mouse Foxp3, and APC-conjugated anti-mouse CD45.2 (104) were purchased from eBioscience. Purified anti-CD73 mAb (clone TY/23; provided by Dr. Linda H. Thompson, Oklahoma Medical Research Foundation), anti-CD4 mAb (GK1.5), anti-CD8 mAb (53-6.7 and 53-5.8), and control Ig (clone MAC4) were produced in house. PE-conjugated MHC (major histocompatibility complex) class I/SINF EK1 tetramers were purchased from Dr Andrew Brooks (University of Melbourne).

In vivo treatments

EG7, MC38-ova, AT-3, and B16F10 tumor cells were injected subcutaneously into syngeneic C57BL/6 wild-type, CD73-deficient, or DEREG mice at the indicated doses. The following mAbs were injected intraperitoneally at 100 μg per dose at the indicated time points: anti-asialoGM1 (Wako Chemicals), anti-CD8, anti-CD4, anti-CD73, and control Ig. B16F10 cells were injected intravenously at the indicated dose and lung metastases were counted under dissecting microscope 2 weeks later after fixation in Bouin’s solution. Where indicated, mice were treated with α,β-methylenadenosine 5′-diphosphate (APCP; Sigma) at 20 mg/kg i.v., anti-CD73 mAb (200 μg i.v.), or control Ig (200 μg i.v.). For bone marrow (BM) reconstitution, wild-type and CD73-deficient mice were irradiated (10 Gy from a 137Cs source) and injected i.v. with 2 × 106 BM cells derived from congenic wild-type or CD73-deficient mice. BM chimera mice were housed in microisolators for 8 weeks before analysis and experimentation.

Treg reconstitution and suppression assay

CD4+ CD25− T cells were isolated from the spleens of wild-type and CD73-deficient mice using a mouse Treg-isolation kit and AutoMACS (Miltenyi) according to the manufacturer’s instructions. Cells were at least 90% pure as determined by flow cytometry. DEREG mice were injected with 1 μg of diptheria toxin (DT) intraperitoneally 3 days prior to intravenous injection of 2 × 105 purified Tregs, and then with 500 ng of DT on days 0 and 7. For suppression assays, accessory CD4+ cells, T effector cells (CD4+ CD25−), and Tregs (CD4+ CD25+) were isolated from spleen cells on an AutoMACS (Miltenyi). Accessory cells were treated with mitomycin C (Sigma) and T effector cells were stained with 5 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Invitrogen). A total of 5 × 10^4 T effector cells were cocultured with 5 × 10^4
accessory cells and unlabeled Treg at different ratios and stimulated with 1 μg/mL anti-CD3 (clone 145-2C11). Responder cell proliferation was assayed by flow cytometry after 96 hours.

Tumor-infiltrating lymphocytes

EG7 tumors were excised, minced with scissors, and incubated 1 hour at 37°C for in PBS containing collagenase type 4 (Worthington Biochemical) and DNase I (Roche). Tumor cell suspensions were passed through a 70-μm cell strainer, washed twice in PBS, and resuspended in PBS 2% serum for flow cytometric analysis. Anti-CD16/32 mAb (clone 2.4G2) was used to block Fc receptors. Flow cytometry was performed on a LSR II (BD Bioscience) and analyzed using the software program FCS Express.

IFN-γ release assays

EG7 cells (10^6) were injected in the footpad of wild-type and CD73-deficient mice and 5 days later, popliteal lymph node cells were harvested, plated in 96 U-well plates (3 x 10^5 cells per well), and restimulated with 1 mg/mL ova protein (Sigma). Cell supernatants were harvested 72 hours later and IFN-γ levels measured by ELISA (eBioscience).

Results

CD73-deficient mice are resistant to primary tumor growth

To investigate the contribution of host-derived CD73 on tumorigenesis, we first compared the growth rate of various syngeneic tumors in wild-type and CD73-deficient mice. MC38-ova, EG7, AT-3, and B16F10 tumor cells were injected subcutaneously into wild-type and CD73-deficient B6 mice and tumor growth monitored over time (data represent means of 5 mice per group ± SE; representative of 3 experiments is shown). B, same as A except mice were injected with 10^6 EG7 tumors cells (data represent means of 6 mice per group ± SE; representative of 2 experiments is shown). C, same as A except mice were injected with 5 x 10^5 AT-3 tumor cells (data represent means of 6 mice per group ± SE). D, same as A except mice were injected with 2 x 10^5 B16F10 tumor cells (data represent means of 5 mice per group ± SE; representative of 2 experiments is shown). *, P < 0.05 by Mann–Whitney test.
CD73-deficient mice toward MC38-ova tumors was dependent on CD8\(^+\) T cells, but independent of CD4\(^+\) T cells or NK cells. Similar results were obtained for EG7 tumors (Supplementary Fig. S2A). Notably, CD73-deficient mice were not significantly resistant to parental MC38 tumors, which lack ova and are thus less immunogenic than MC38-ova tumors. The increased immunogenicity of MC38-ova tumors is evidenced by their ability to generate endogenous CD8\(^+\) T-cell–mediated control, which is not observed for parental MC38 tumors (Supplementary Fig. S2B and C). Our data thus suggest that host-derived CD73 is important in suppressing existing CD8\(^+\) T-cell–mediated antitumor responses.

We next compared the systemic frequency, tumor infiltration, and function of antigen-specific CD8\(^+\) T cells in CD73-deficient and wild-type mice. Flow cytometric analysis revealed that tumor-bearing CD73-deficient mice had an increase proportion of tumor-specific CD8\(^+\) T cells in peripheral blood (Fig. 2B). Tumor-specific CD8\(^+\) T cells were also found more abundantly infiltrated in the tumors of CD73-deficient mice (Fig. 2C). In contrast, tumor infiltration by Foxp3\(^+\) CD4\(^+\) Tregs was similar in CD73-deficient and wild-type mice (Fig. 2D). Consistent with an increased CD8\(^+\) T-cell–mediated antitumor immune response, antigenic restimulation of tumor-draining lymph node cells isolated from
tumor-bearing CD73-deficient mice generated significantly greater IFN-γ production compared with restimulation of wild-type tumor-draining lymph node cells (Fig. 2E).

CD73 is expressed on hematopoietic and nonhematopoietic cells. To further assess the contribution of CD73 expression on hematopoietic versus nonhematopoietic cells, we generated CD73 BM chimera mice. BM reconstitution was confirmed by flow cytometry analysis 8 weeks after BM cell injection and before tumor cell inoculation (Supplementary Fig. S3). As shown in Figure 2F, CD73 expression on either hematopoietic or nonhematopoietic cells significantly enhanced MC38-ova tumor growth, whereas CD73 expression in both hematopoietic and nonhematopoietic cells was most effective. Our data thus suggest that hematopoietic and nonhematopoietic expression of CD73 promotes tumor immune escape.

CD73 expression on Tregs is critical for Treg-mediated tumor growth

In hematopoietic cells, CD73 is most highly expressed on activated Foxp3+ CD4+ CD25+ Tregs (23). We thus investigated the specific role of CD73 expression on Foxp3+ Tregs during tumor growth. Foxp3+ Tregs develop normally in CD73-deficient mice, but their immunosuppressive function is compromised (Fig. 3A). This is consistent with other studies that demonstrated that Tregs partly rely on CD73-mediated production of extracellular adenosine for immunosuppression (11, 23, 28, 29). Nevertheless, the importance of CD73 in Treg-mediated tumor growth remains unknown. To assess the role of CD73 expression by Tregs during tumor growth, we first compared the tumor-promoting effects of Tregs in wild-type and CD73-deficient mice. As shown in Figure 3B, anti-folate...
receptor 4 (FR4)-mediated Treg depletion in wild-type mice (30) significantly abrogated MC38-ova tumor growth. In contrast, Treg depletion in CD73-deficient mice had no effect. This suggested that Tregs in CD73-deficient mice failed to promote tumor growth. We hypothesized that this was due to the fact that Tregs and host-derived CD73 had overlapping protumorigenic effects; that is, CD73 expression on Tregs was important for tumor growth. To directly assess this, we used transgenic DEREG mice, which express a DT receptor under Foxp3 promoter that allows for the complete depletion of Foxp3+ Tregs after DT injection (30, 31). As shown in Figure 3B and C, neutralization of CD73 induced comparable anti-tumor effects to Treg depletion with anti-FR4 mAb, and CD73 neutralization was slightly less effective than total Treg ablation with DT. Taken together, these results were consistent with the presence of overlapping protumorigenic effects between Tregs and host-derived CD73.

We next performed adoptive cell reconstitution of Treg-depleted DEREG mice with purified CD4+CD25+ Tregs isolated from wild-type or CD73-deficient mice. Flow cytometric analysis confirmed adoptive cell reconstitution (Supplementary Fig. S5). As shown in Figure 3D, only DEREG mice reconstituted with wild-type Tregs, but not DEREG mice reconstituted with CD73-deficient Tregs, could effectively support MC38-ova tumor growth. Taken together, our data demonstrated that in the MC38-ova tumor model, part of the protumorigenic effect of Tregs is dependent on CD73 expression by Tregs.

CD73-deficient mice are resistant to B16F10 lung metastasis

We next investigated the susceptibility of CD73-deficient mice to develop experimental lung metastases following intravenous injection of CD73-negative B16F10 melanoma cells. As shown in Figure 4A, CD73-deficient mice were significantly resistant to the development of B16F10 lung metastases. Accordingly, CD73-deficient mice developed 3- to 4-fold less metastatic lung nodules after intravenous injection of B16F10 cells. Unexpectedly, this resistance was maintained in NK-cell–depleted or T cell–depleted CD73-deficient mice (Fig. 4A). This suggests that host-derived CD73 promotes B16F10 lung metastasis independently of its immunosuppressive effect on NK cells. To further assess the mechanism of resistance of CD73-deficient mice against experimental metastasis, BM chimera mice were used. As shown in Figure 4B, the lack of CD73 expression on nonhematopoietic cells was largely responsible for the resistance of CD73-deficient mice against experimental lung metastasis. Our data suggest that CD73 expression on nonhematopoietic essentially endothelial cells enhances the metastatic potential of circulating tumor cells.

CD73-targeted therapy inhibits the growth and metastasis of CD73-negative tumors

We further assessed the therapeutic activity of CD73-targeted therapy against CD73-negative tumors. Consistent with the therapeutic effect observed against MC38-ova tumors (Fig. 3C), treatment of EG7 tumors with the CD73 inhibitor APCP also induced significant antitumor effect (Fig. 5A). We then assessed the therapeutic activity of CD73 inhibition on the development of B16F10 lung metastases after intravenous tumor cell injection. As shown in Figure 5B, inhibition of CD73 with APCP or anti-CD73 mAb significantly suppressed the development of B16F10 lung metastases in wild-type mice. Notably, only when CD73 was blocked prior to B16F10 cell injection, and not after B16F10 cell injection, was the development of lung metastases suppressed. This further supports the concept that CD73 expression on endothelial cells promotes tumor
cell metastasis. Taken together, our data suggest that inhibition of host-derived CD73 can effectively suppress tumor growth as well as the metastatic potential of circulating tumor cells.

Discussion

We have previously shown that CD73 expression on breast cancer cells significantly inhibits adaptive tumor immunosurveillance (14). The objective of this study was now to investigate the role of host-derived CD73 on tumorigenesis. We here demonstrated that (i) CD73-deficient mice are resistant to the development of immunogenic tumors in a CD8+ T-cell–dependent manner; (ii) hematopoietic and nonhematopoietic expression of CD73 each promote tumor immune escape in a non-redundant manner; (iii) CD73 expression on Foxp3+ Tregs mediates part of the pro-tumorigenic effect of Tregs; (iv) nonhematopoietic expression of CD73, presumably on endothelial cells, enhances metastasis of circulating tumor cells to the lungs; and (v) targeted inhibition of CD73 suppresses the subcutaneous growth and the metastasis of CD73-negative tumor cells.

An important aspect of this study was to use Treg reconstitution of DEREG mice, which express the DT receptor under Foxp3 promoter, to directly demonstrate the importance of CD73 expression on Tregs during tumorigenesis. At least in the MC38-ova tumor model, extracellular adenosine production by Tregs appears to be an essential component of Treg-mediated immunosuppression. To our knowledge, this is the first time such reconstitution experiments have been used to reveal a key functional molecule on Tregs. Many mechanisms are involved in Treg-mediated immunosuppression. Our study defines CD73 as an important Treg immunosuppressive factor for promoting tumor growth. Future comparative studies with other important Treg molecules will now be of interest.

In lymphocytes, only Foxp3+ Tregs are endowed with the complete catalytic machinery able to produce extracellular adenosine. Accordingly, Foxp3+ Tregs constitutively express CD39 (which hydrolyses ATP and ADP) and CD73 (which hydrolyses AMP into adenosine). CD39/CD73 coexpression on Tregs has been shown to be maintained by IL-2 (interleukin-2; ref. 32) and upregulated upon activation (23). CD39/CD73 coexpression on Tregs converts proinflammatory extracellular ATP into the potent immunosuppressor adenosine. Tregs have previously been shown to partly rely on the enzymatic activity of CD39 for immunosuppression. Indeed, Deaglio and colleagues (11) demonstrated that CD39-deficient Tregs have impaired immunosuppressive function in vitro and fail to block skin allograft rejection in vivo. The data presented here further demonstrate the importance of CD73 on Tregs and suggest that CD73 and CD39 have nonredundant immunosuppressive effects.

The adenosine-mediated immunosuppressive function of Tregs has been shown to be essentially dependent on the activation of A2A adenosine receptor of effector T cells. While extracellular adenosine activates A2A adenosine receptors on effector T cells, Tregs can also accumulate cyclic AMP (cAMP) and transfer cAMP to effector T cells via gap junctions (33). Thus, extracellular adenosine production by Tregs could theoretically suppress effector T cells via 2 nonexclusive mechanisms: via activation of A2A adenosine receptors on effector T cells and/or via activation of A2A adenosine receptors on Tregs followed by transfer of cAMP via gap junctions.
A recent study by Sun and colleagues (34) investigated the role of host-derived CD39 on tumor growth and tumor cell metastasis. Consistent with our study, Sun and colleagues (34) demonstrated that B16F10 metastasis was strongly inhibited in mice with CD39-deficient nonhematopoietic cells. However, B16F10 metastasis was also inhibited in mice with CD39-deficient hematopoietic cells, in contrast to what we observed for CD73. Sun and colleagues (34) demonstrated that Tregs inhibit NK-cell–mediated antitumor functions in a mechanism dependent on CD39 expression. In contrast, we showed that host-derived CD73 promotes B16F10 metastasis independently of its effect on NK cells. Since both studies were performed with the same tumor model, this suggests the presence of distinct nonredundant prometastatic effects for host-derived CD39 and host-derived CD73. Further studies should clarify the mechanism by which nonhematopoietic expression of CD73 promotes lung metastasis. Our hypothesis is that endothelial CD73 expression may promote transendothelial migration of tumor cells. While endothelial CD73 can decrease leukocyte attachment and migration into draining lymph nodes (22), it can also promote endothelial extravasation in pathologic conditions. Accordingly, decreased T-cell extravasation was recently suggested as the cause of resistance of CD73-deficient mice to experimental autoimmune encephalomyelitis (35). Through the production of extracellular adenosine and the activation of adenosine receptors on endothelial cells, Mills and colleagues (33) suggested that endothelial CD73 can enhance expression of adhesion molecules and transendothelial migration.

In conclusion, whereas the immunosuppressive effects of tumorderived CD73 have recently been studied, the effects of host-derived CD73 on antitumor immunity have not been addressed. We here demonstrated an important role for CD73 expression on Tregs for the suppression of adaptive antitumor immune responses. Together with other studies, this suggests that the combined activity of CD39 and CD73 is a key component of Treg-mediated suppression. We also demonstrated an important role for CD73 expression on nonhematopoietic cells, presumably endothelial cells, on the metastatic potential of circulating tumor cells. Finally, we demonstrated that CD73 inhibition significantly inhibits the growth and metastatic potential of CD73-negative tumor cells. We propose that CD73 may be targeted at multiple levels to induce antitumor effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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